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Photoreceptors of mouse retinas possess D_4 receptors coupled to adenylate cyclase

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ABSTRACT **In the mouse, the light-sensitive pool of cAMP can be eliminated in the dark by application of the dopamine D_2-like receptor agonists LY 171555 (quinpirole), (+)-N0437 {2-[*N*-(*n*-propyl)-*N*-2-(thienylethylamino)-5-hydroxytetralin]}, or (+)-3-PPP [3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride]. The rank-order affinity of the ability of the D_2-like antagonists to block the action of LY 171555 matched that of the rat D_4 receptor. Reverse transcription of retina mRNA followed by DNA amplification using D_4-specific nucleotides demonstrates the presence of D_4 mRNA in retina. *In situ* hybridization studies using D_4-specific digoxygenin-labeled oligonucleotides or ^{35}S-labeled UTP RNA probes demonstrate the presence of D_4 mRNA in the photoreceptor cell layer and in the inner nuclear and ganglion cell layers. The modulation by D_4 ligands of the dark level of light-sensitive cAMP in photoreceptors demonstrates the physiological coupling of the D_4 receptor subtype.**

The identification and characterization of dopamine receptors represents an issue of wide interest in neurobiology, particularly in light of the recent work (43) demonstrating the multiplicity of types of dopamine receptor. When cloned and expressed in transfected cell lines, these subtypes reveal distinct pharmacological differences when compared in the same cell background (1–8). Thus, in mouse fibroblasts, the human D_4 receptor has a 10- to 15-fold higher affinity for quinpirole (LY 171555), a 2-fold lower affinity for sulpiride, and a 5- to 15-fold higher affinity for clozapine than does the D_2 receptor (7). Similar results were found for the rat D_4 receptor (9). The D_3 receptor has a distinct D_2-like binding profile in Chinese hamster ovary cells but does not appear to be physiologically coupled to a guanine nucleotide binding protein (6). The functional significance of binding differences in transfected heterologous cell types is unclear.

The retina, a model central nervous system tissue, is well suited for studies of dopaminergic pathways, and previous studies on the retina have implicated potential roles for dopamine in a number of processes. This central nervous system division contains biosynthetic enzymes for dopamine production, and classically defined D_1 and D_2 dopamine receptors that modulate levels of cAMP. For example, the photoreceptor pool of cAMP is sensitive to either light or dopamine (10) and can be studied selectively by using incubation medium containing 10 mM glutamate or aspartate. Such media eliminate the light-evoked responses of neurons postsynaptic to photoreceptors (11–18), allowing the identification of transmitters that in the dark can alter the size of the light-sensitive pool of cAMP through their action on membrane receptors on these cells. Recent pharmacological experiments (10) examining regulation of the size of this cAMP pool found evidence for its modulation by a receptor distinct from the classic D_2 receptor.

Previous autoradiographic studies suggested that D_2-like receptors were present throughout the outer nuclear layer (19–22). However, studies using D_2-specific mRNA probes failed to observe D_2 mRNA within the photoreceptor layer of the rat (23), while in primate photoreceptors, one failed (24) but another succeeded (25). Here we demonstrate that mouse retinal photoreceptors possess D_4 receptors linked to the inhibition of adenylate cyclase.

MATERIALS AND METHODS

Animals, Retinal Isolations, and Incubations. The retinas in most of these experiments were from mice of the C57BL/6J strain, purchased from The Jackson Laboratories and maintained under a 12-hr dark/12-hr light cycle. Animals were taken from the light phase and dark-adapted for at least 1 hr before use. Some special mice were bred from mutant stock from the above source to obtain genetically homozygous photoreceptor-deficient light-eared (*rd le/rd le*) animals with a C57BL/6J background and normal appearing heterozygous (*rd le/++*) controls. As the recessive *rd* and *le* genes are closely linked, homozygous totally rodless and largely cone-deficient animals are identified by light-colored ears and tails. All retinas were from animals of 90–120 days of age.

Dark-adapted retinas were isolated as described (26) in ice-cold Earle's medium under narrow-band dim red illumination. The isolation medium was Earle's saline containing 115 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 26 mM $NaHCO_3$, 0.9 mM NaH_2PO_4, and 5.5 mM dextrose (pH 7.4 in an atmosphere of 5% CO_2/95% O_2). In our incubations, 10 mM NaCl was replaced by 10 mM sodium glutamate and the medium also contained 1 mM 3-isobutyl-1-methylxanthine, which elevated cAMP but only partially blocked the ability of light to reduce cGMP (10). White light delivered during incubations was infrared-filtered and had an intensity of 1600 lm/m^2 at the retinas.

Cyclic Nucleotide Assays. The retinas were frozen in liquid nitrogen after incubation and then homogenized in 10% (wt/vol) trichloroacetic acid. The homogenate was centrifuged, and the supernatant was ether-washed, dried-down, and reconstituted in 50 mM sodium acetate, pH 5.8. The cAMP was acetylated and measured by radioimmunoassay (27, 28). Protein content was determined by the method of Lowry *et al.* (29).

Pharmacology. The basic incubation medium contained Earle's saline, 10 mM glutamate, and 1 mM 3-isobutyl-1-methylxanthine. All control or experimental incubations were for 9 min in the dark or for 3 min in the dark followed by 6 min in bright light. A control set of six retinas from three dark-adapted mice were individually incubated in 400 μl of the basic Earle's medium. A second control set differed only in that the medium additionally contained the D_2 agonist LY

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171555 at 1 μM, a level that totally eliminated the light-sensitive pool. A third set of controls lacked LY 171555 but contained the highest concentration of the antagonist under investigation. The subsequent dark- or dark–light-incubated sets contained both 1 μM LY 171555 and one of a graded series of concentrations of the antagonist under investigation.

Oligodeoxynucleotides. Oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer. Oligonucleotides were derived from the rat D_2 receptor DNA (5) and the rat D_4 receptor gene (9). The D_2 receptor included the following oligonucleotides: oD2-224, 5′-TGGTGGGATGGATCAGGGAGAGTGAG-3′ [complementary to nucleotides (nt) 832–857]; oD2-232, 5′-TACATCGTCCTCCGGAAGCGCCGGAA-3′ (identical with nt 637–662); oD2-218, 5′-TTTCCAGCTCCTGAGCTC-3′ (complementary to nt 877–894); oD2-560, 5′-GCTGATCTCCCTCTACTCTCCAATCCACTC-3′ (identical with nt 2087–2116); oD2-561, 5′-ATGGTGTTGGCAGGGAACTCGCTCGTTTGC-3′ (complementary to oD2-560). Primers oD2-224, -232, -560, and -561 have a single mismatch with the analogous mouse D_2 sequence (30); primer oD2-218 has no mismatches. Primers derived from the rat D_4 sequence (9) included the following oligonucleotides: oD4-466, 5′-CAGACACCGACCAACTAC-3′ (identical with nt 187–204); oD4-465, 5′-TTGAAGATGGAGGGGGTG-3′ (complementary to nt 342–359); oD4-474, 5′-TGACACCCTCATGGCCAT-3′ (identical with nt 309–326). Oligonucleotides used for *in situ* hybridization included: oD4-562, 5′-ATGGTGTTGGCAGGGAACTCGCTCGTTTGC-3′ (identical with nt 124–153); oD4-563, 5′-GCAAACGAGCGAGTTCCCTGCCAACACCAT-3′ (complementary to the same nucleotides).

Polymerase Chain Reaction (PCR). For amplification of RNA, 1 μg of total retinal RNA (31) was reversed-transcribed (32) with either oD2-224 or oD4-465. Second-strand synthesis and further amplification was done using 250 ng of cDNA and 5 pmol each of primer sets oD2-232/224 and oD4-465/466. Temperatures for D_2 amplification were 93°C (1 min), 61°C (1 min), and 72°C (1 min) for 30 cycles. Full-length rat $D_{2\text{-}444}$ and $D_{2\text{-}415}$ cDNAs were amplified at the same time as controls. Temperatures and times were the same for the D_4 mRNA except that the annealing temperature was 50°C. Linearity of the reaction was verified using our published protocols (30, 33). PCR products were analyzed by PAGE on a 5% gel, transferred to nylon, and probed with the internal amplification primers oD2-218 and oD4-474, respectively, prior to autoradiography.

***In Situ* Hybridization Using Digoxygenin-Oligonucleotides.** The eyecups or retinas of C57BL/6J mice were isolated in white light in ice-cold medium, frozen in OTC compound (Lab-Tek) and then sectioned in a cryostat at −25°C, and slices were mounted on subbed slides that were stored in a deep freeze at −80°C or in crushed frozen CO_2. Sense and antisense D_4 oligonucleotide probes were end-labeled with digoxygenin-derivitized dUTP according to the manufacturer's protocols (Boehringer Mannheim). Hybridization conditions were essentially as described (34). The probe concentration was 35 ng/ml and hybridization was overnight at 37°C. The final stringency wash was 0.5× SSC at 22°C for 30 min.

***In Situ* Hybridization Using ^{35}S-labeled RNA Probes.** Alternatively, adjacent retinal sections were hybridized with one of two ^{35}S-labeled UTP-labeled RNA probes: (*i*) a 495-base-

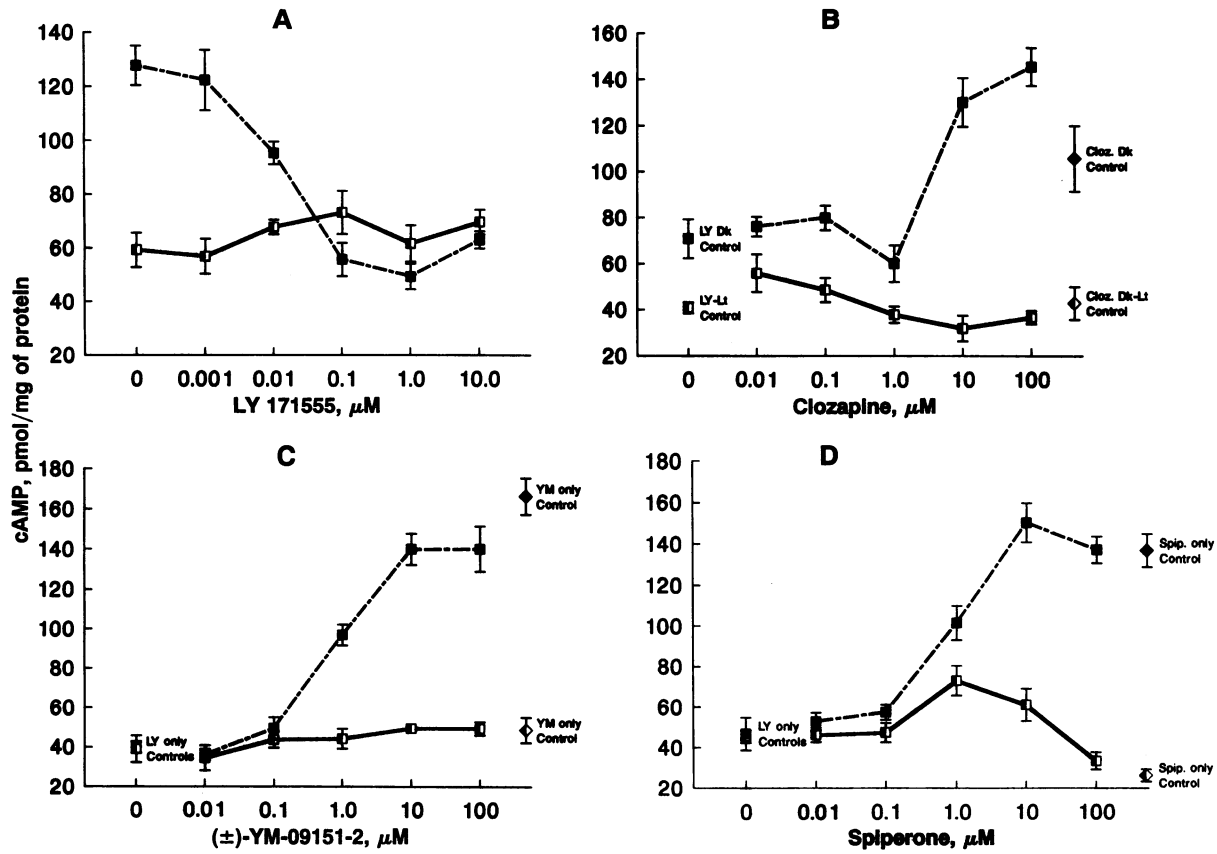

FIG. 1. (*A*) Dose–response curve for the action of LY 171555 on the retinal level of cAMP. (*B–D*) Effects of increasing concentrations of D_2-like dopamine antagonists on the ability of LY 171555 to reduce the retinal level of cAMP. Except for controls, medium contained the indicated level of clozapine, (±)-YM-09151-2, or spiperone (plus 50 μM ketanserin). Controls lacked either LY 171555 or any antagonist. Incubations were for 9 min in the dark (solid squares) or for 3 min in the dark followed by 6 min in the light (solid/open squares). All media contained 1 mM 3-isobutyl-1-methylxanthine and 10 mM glutamate. LY, LY 171555; Dk, dark; Lt, light; Cloz, clozapine; YM, YM-09151-2; Spip, spiperone.

pair RNA probe corresponding to a *Sac* I–*Bgl* II fragment of the rat D_2 receptor (5), which would recognize both $D_{2\text{-}444}$ and $D_{2\text{-}415}$ and (*ii*) a 420-base-pair RNA probe to human D_4 corresponding to the 3′ untranslated region and transmembrane domains VI and VII (7). Slides had both eyecup and retinal sections and were processed as described (35). Probe preparation, hybridization, and wash conditions were as described (35), except that slides were treated with RNase for only 30 min and given a final wash in 0.1× SSC at 65°C for 1 hr. To assess the specificity, both sense strand and RNase-pretreated controls were employed (35, 36). Slides were stained for cell nuclei and then examined by phase-contrast and dark-field microscopy.

Sources of Pharmacological Agents. The D_2 (also D_3 and D_4) agonist LY 171555 was a generous gift from Eli Lilly; the (+)- and (−)-enantiomers of N0437 {2-[*N*-(*n*-propyl)-*N*-2-(thienylethylamino)-5-hydroxytetralin], also termed (+)-N-0924 and (−)-N-0923} were generous gifts from Whitby (Irvine, CA). The D_2 agonists (+)- and (−)-3-PPP [3-(3-hydroxyphenyl)-*N*-propylpyridine hydrochloride] and the antagonists (+)-butaclamol and (±)-YM-09151-2 {[*N*-(*2RS,3RS*)-1-benzyl-2-methyl-3-pyrrolidinyl]-5-chloro-2-methoxy-4-methylaminobenzamide} were obtained from Research Biochemicals (Natick, MA) the latter as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract 278-90-0007(BS). Clozapine was a generous gift from John Cernansky (Washington University School of Medicine, St. Louis).

RESULTS

As reported (10) when dark-adapted retinas were incubated in 3-isobutyl-1-methylxanthine–glutamate medium in either the dark or in darkness followed by light, the D_2 agonists LY 171555 or (+)-3-PPP reduced the dark level of cAMP to that seen in bright light. The same has been observed (44) with the agonist (+)-N0437. The inactive enantiomers (−)-N0437 and (−)-3-PPP had no effect. The half-maximal effective dose for LY 171555 was ≈10 nM (Fig. 1*A*). This agent, at 0.1 μM or greater, reduced the dark level of cAMP to that seen in light. As seen in Fig. 1 *B–D*, the simultaneous presence of the D_2 agonist LY 171555 at 1 μM and the D_2 antagonists (±)-YM-09151-2 (a class 3 substituted benzamide) at 1 μM, clozapine at 1 μM, or spiperone at 1 μM (in the presence of ketanserin) blocked the effect of 1 μM LY 171555. In contrast, when similarly employed, 1 nM–10 μM (+)-butaclamol or the type 2 substituted benzamides (−)-sulpiride from 10 nM to 500 μM or (−)-eticlopride from 10 nM to 1 mM exhibited no antagonism whatsoever to the action of 1 μM LY 171555. When LY 171555 was employed at 0.1 μM, 100 μM eticlopride significantly antagonized the action of the agonist (data not shown). These results suggest that the D_2-like response in dark-adapted mouse photoreceptors is not due to a "traditional" D_2 receptor. The pharmacological response parallels that reported for the human (7) and rat (9) D_4 receptor.

To confirm the hypothesis that the photoreceptors contained D_4-like receptors, we tested whether these cells expressed D_4 mRNA. We compared photoreceptor containing (*rd le/++*) mouse retinas with totally rod-deficient virtually cone-deficient (*rd le/rd le*) mouse retinas for D_2 and D_4 mRNA. As shown in Fig. 2, total RNA prepared from retinas with or without photoreceptors contained $D_{2\text{-}444}$, $D_{2\text{-}415}$, and D_4 transcripts. When normalized to 18S rRNA, there appeared to be approximately equal amounts of the D_2 [$D_{2\text{-}444}$ and $D_{2\text{-}415}$] and D_4 mRNAs expressed (data not shown). These results and our *in situ* hybridization studies (see below) indicate that D_4-like receptors are present in retinal tissue but that they are not unique to photoreceptors.

The cellular distribution of D_4 and D_2 mRNA was further characterized by *in situ* hybridization. We used digoxygenin-labeled oligonucleotides derived from the rat D_4 (9), D_2 (33), and D_3 (6) genes that would hybridize with the analogous mouse sequences. Fig. 3 demonstrates D_4 labeling at the inner segment level of the photoreceptors, the inner nuclear

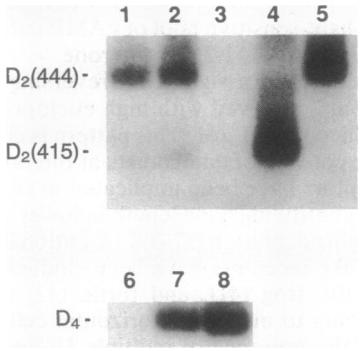

FIG. 2. Expression of D_2 and D_4 mRNA in mouse retinas. Total RNA was prepared and equivalent amounts of RNA from *rd le/++* or *rd le/rd le* mouse retinas were reverse-transcribed and amplified with either D_2- or D_4-specific primers. Lanes 1–5 are reaction products observed with the D_2 primer set. Lanes: 1, *rd le/++*; 2, *rd le/rd le*; 3, PCR control; 4, $D_{2\text{-}415}$ cDNA control; 5, $D_{2\text{-}444}$ cDNA control. Lanes 6–8 show fragments amplified with the D_4 primer set. Lanes: 6, D_4-specific PCR mixture control; 7, *rd le/++*; 8, *rd le/rd le*.

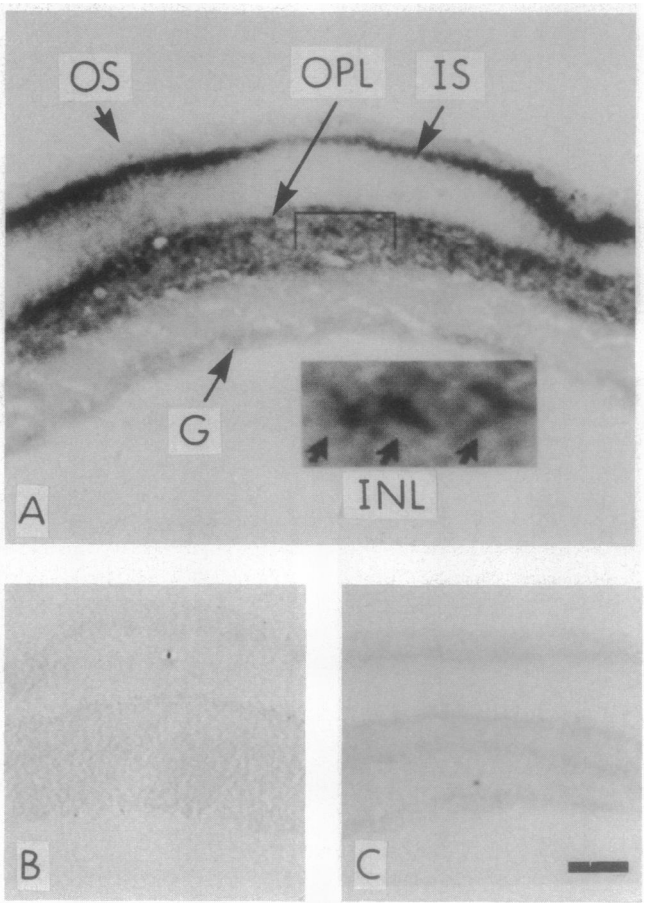

FIG. 3. Retinal D_4 receptor immunolocalizations employing digoxygenin-labeled D_4 oligonucleotide probes and controls. The probe was antisense in *A*, oligonucleotide-free in *B*, and sense in *C*. (Bar = 50 μm.) The retinal levels indicated are the outer segment (OS) and inner segment (IS) of the photoreceptor, the outer plexiform layer (OPL), the inner nuclear layer (INL) (enlarged ×2.5 in box), and the ganglion cell level (G). Images from eosin-counterstained originals were computer enhanced with an IMAGE-1 system.

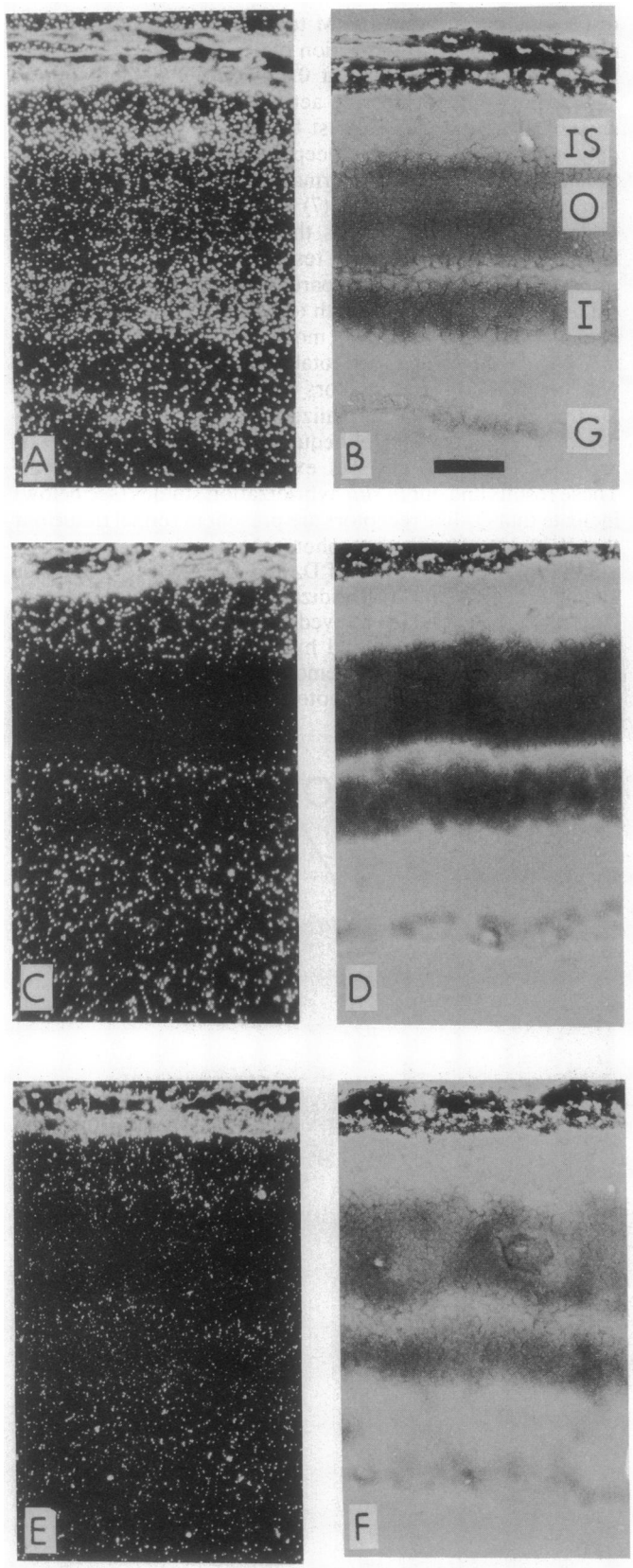

FIG. 4. Paired views of identical sections illustrating radiolabeling by dark-field microscopy (*A*, *C*, and *E*) and retinal layers by phase-contrast microscopy (*B*, *D*, and *F*). (*A*) Hybridization with a ^{35}S-labeled UTP antisense RNA probe. (*C*) Sense probe was employed. (*E*) RNase treatment preceded exposure to an antisense probe. The retinal nuclear layers shown are the outer nuclear layer of photoreceptors (O), with silver grains in *B* concentrated at their inner segment level (IS), the inner nuclear layer (I), and the layer of ganglion cells (G). (Bar = 36 μm.)

layer, and the ganglion cell layer (three of three experiments). The photoreceptor inner segment layer is uniformly stained. Cell staining in the inner nuclear layer is heterogenous where some cells are more intensely stained than others (Fig. 3*A*). In data not shown, with D_2 probes labeling was typically confined to the inner nuclear layer and ganglion cell layers and D_3 probes yielded no labeling whatsoever.

To confirm and extend these findings, we used ^{35}S-labeled UTP-labeled RNA probes for D_2 and D_4. By using a ^{35}S-labeled UTP-labeled human D_4 RNA probe, labeling was detected not only in photoreceptors (inner segment level) but also in the inner nuclear layer and ganglion cell layers of the retina (Fig. 4). In contrast, a D_2 RNA probe labeled the inner nuclear layer and ganglion cell layers. In two of six experiments for D_2, using either methodology, labeling in the photoreceptor layer was seen only under conditions of reduced stringency.

DISCUSSION

To date, no signal transduction mechanism for the D_4 receptor has been reported. This study documents the presence of D_4 receptors in mouse retina and links D_4 pharmacology to the modulation of the photoreceptor light-sensitive pool of cAMP. Therefore, like the D_2 receptor, stimulation of D_4 binding sites appears to be linked to adenylate cyclase. Previous D_2 localization studies in photoreceptors have been equivocal. Although the possibility exists that some D_2 mRNA is expressed in the photoreceptor layer (at least in human retina; ref. 25), our pharmacological studies are not consistent with "traditional" D_2-like responses nor do our *in situ* studies provide compelling data for the presence of D_2 receptors in the mouse photoreceptor layer. We conclude that under these experimental conditions the dopamine modulation of photoreceptor cAMP is mediated by D_4 receptors.

Pharmacologically, while all of the D_2-like receptors bind the classic D_2-selective ligands, rank-order potencies vary between receptor subtypes. Van Tol *et al.* (7) reported the human D_4 receptor had a 10- to 15-fold higher affinity for the atypical neuroleptic clozapine than did the D_2 receptor. The rat D_4 receptor has only a 2- to 3-fold increased affinity for this ligand (9). Additionally, the rat D_4 receptor has an $\approx$1000-fold lower affinity for eticlopride and an $\approx$100-fold lower affinity for (+)-butaclamol (L. Tang, R.T., and K.O., unpublished data).

In whole tissue, the rank order of ligands acting on second messengers may be influenced by their diffusion into tissue as affected by molecular charge, size, and hydrophobicity. In the current study, the rank-order ability of ligands to reverse the loss of the light-sensitive pool of cAMP induced by 1 μM LY 171555 was YM-09151-2 = spiperone >> clozapine. In mouse photoreceptors, a significant reversing effect of eticlopride was only observed with high eticlopride levels and LY 171555 reduced to 0.1 μM. This pattern is consistent with the rank-order potency of antagonists at the rat D_4 receptor.

D_2-like receptors have been implicated in signal transduction pathways controlling cone length in lower vertebrates as a function of light adaptation (37–39). Additionally, dopamine effects via D_2-like receptors have been studied in horizontal cells of fish (40), frog (41), and turtle (42) retinas where dopamine appears to modulate horizontal cell actions. The recognition of the presence of multiple D_1 and D_2 receptor subtypes clearly requires a reevaluation of previous physiological and/or pharmacological studies of retinas (and other central nervous system regions) as to subtype identity and significance.

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